American Journal of Hematology 52:178–183 (1996)

Possible Role of Tumor Necrosis Factor-Alpha in Erythropoietic Suppression by Endotoxin and Granulocyte/Macrophage Colony-Stimulating Factor

K.B. Udupa and B.G. Sharma

Geriatric Research, Education and Clinical Center, V.A. Medical Center and Departments of Medicine, Physiology, and Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas (K.B.U.); R.W. Johnson Pharmaceutical Research Institute, Raritan, New Jersey (B.G.S.)

Injection of bacterial endotoxin or granulocyte/macrophage colony-stimulating factor (GM-CSF) into exhypoxic polycythemic mice simultaneously with erythropoietin (EPO) suppressed erythroid cell formation, as monitored by ^{59}Fe incorporation into circulating red blood cells. This effect was dose-dependent and time-dependent. GM-CSF did not inhibit erythroid cell formation directly, as the antibody to the GM-CSF did not neutralize the effect of endotoxin, the inducer of GM-CSF. The suppression of both agents could be partially corrected by prior injection of a monoclonal antibody to tumor necrosis factor α (anti-TNFα). These results indicate that the suppression of EPO-induced erythroid cell formation by endotoxin and GM-CSF was due in part to the production of TNFα.

© 1996 Wiley-Liss, Inc.

Key words: erythropoietin, endotoxin, granulocyte/macrophage colony-stimulating factor, tumor necrosis factor α, mouse

INTRODUCTION

Bacterial endotoxin injection into mice has been shown to reduce the red blood cell (RBC) progenitors in the bone marrow, while those in the spleen are increased [1,2]. We showed earlier that endotoxin decreases the response of exhypoxic polycythemic mice to erythropoietin (EPO), as assessed by reticulocyte count, ^{59}Fe incorporation into RBC, and marrow ^{59}Fe uptake [3]. Injection of this toxin into animals elevates the levels of several cytokines and other factors, including granulocyte/macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNFα), and interleukin-1α (IL-1α) [4–6]. The observed effect of endotoxin upon erythropoiesis can be due to any of these, acting alone or in combination. With the advancement of recombinant technology, large quantities of these cytokines, colony-stimulating factors, and their antibodies can be prepared in pure form. Using some of these recombinant products we showed that IL-1α, TNFα, and GM-CSF can negatively influence erythroid colony development in vitro [7]. This study also indicated that in vitro suppression of erythroid colony development is a result of the production of TNFα, by IL-1α and/or GM-CSF [7]. In vivo neutralization of TNFα is also an important step for the survival of mice following an injection of a lethal dose of endotoxin [8]. The relationship of this protection to erythropoietic suppression is yet to be determined.

We have undertaken studies to identify the factors that suppress erythroid cell proliferation following endotoxin and recombinant murine GM-CSF injections. We have examined this suppression in exhypoxic polycythemic mice, in which a wave of erythroid cell formation was initiated by a single injection of EPO. The primary effect of the administration of both endotoxin and GM-CSF was to induce TNFα production, which in turn suppressed erythroid cell proliferation.

MATERIALS AND METHODS

BDF_1 female mice, age 7–8 weeks, obtained from Charles River Laboratory (Wilmington, MA), were made

Received for publication June 2, 1995; accepted February 7, 1996.

Address reprint requests to K.B. Udupa, Ph.D., V.A. Medical Center, 4300 West Seventh Street, Little Rock, AR 72205.

© 1996 Wiley-Liss, Inc.

polycythemic employing standard techniques of 18-hr exposure to 0.4 atmospheric pressure daily for 14 days in specially constructed chambers [9]. This treatment increased the hematocrit of the mice from the normal value of 50% to 75–80%. After this treatment the mice were removed from the hypoxic chamber and kept at ambient pressure. At various intervals they were injected with GM-CSF, endotoxin, and/or anti-TNFα, as indicated in Results. As a control for anti-TNFα injection, an equivalent quantity of hamster IgG was injected at similar intervals. All of these mice were injected with EPO on day 3 after the termination of hypoxic treatment, and with ^{59}Fe on day 5. Two days later (day 7), the mice were anaesthetized with CO_2, and blood was collected by heart puncture. An aliquot of the blood was pipetted into a plastic tube for determination of ^{59}Fe incorporation and for hematocrit determination. Polycythemic mice with hematocrit <58% were not included in the calculation to ensure that the iron incorporation into RBCs was only due to the erythroid cells produced by EPO. The ^{59}Fe count in the measured aliquot of blood was converted into total circulating RBC incorporation by assuming blood volumes to be 8% of the body weight of polycythemic mice.

GM-CSF and its antibody were procured from Upstate Biotechnology, Inc. (Lake Placid, NY), and anti-TNFα was purchased from Genzyme (Cambridge, MA). All these materials were certified by the manufactures to be >98% pure. According to them, the endotoxin contents of GM-CSF and its antibody were 0.01 ng/μg, and no endotoxin was detectable in the anti-TNFα preparation. Human recombinant EPO was kindly provided by the R.W. Johnson Pharmaceutical Research Institute (Raritan, NJ). Endotoxin (lipopolysaccharide B, *S. typhosa*) was procured from Difco Laboratories (Detroit, MI).

Results are reported as mean ± standard error of the mean (SEM). Data sets were compared using Student's *t*-test to identify significant differences; $P < 0.5$ was considered significant.

RESULTS

Injection of bacterial endotoxin into normal mice reduces their erythropoiesis, and this effect is dose-dependent [10,11]. In the case of exhypoxic polycythemic mice, inhibition of erythroid cell formation by endotoxin depends not only on the dose but also on the time of its injection with respect to EPO. We showed earlier that a simultaneous injection of endotoxin and EPO causes maximum suppression of the incorporation of injected radioactive iron into RBCs [3]. In the present study we determined that simultaneous injection of 0.05 μg endotoxin together with 0.1 UEPO produced significant inhibition of iron incorporation ($P < 0.05$) (Fig. 1).

It is well-known that injection of endotoxin induces the production of GM-CSF, and its level in the circulation

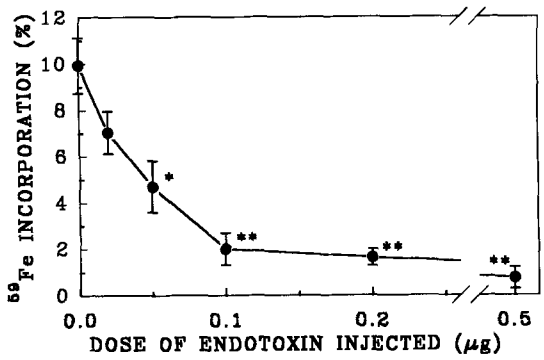

Fig. 1. ^{59}Fe incorporation into circulating RBCs (% of injected dose) of exhypoxic polycythemic mice, when 0.025–0.5 μg endotoxin were injected simultaneously with 0.1 U EPO. Values are mean ± SEM of 6 mice per group (values were collected in at least two separate experiments). *$P < 0.05$; **$P < 0.01$.

is elevated within 3 hr [12]. In order to study whether this observed effect on erythropoiesis was due to the elevation of GM-CSF, 1 μg of murine recombinant GM-CSF (containing 0.01 ng endotoxin) was injected into polycythemic mice, together with 0.1 U EPO. This treatment decreased the iron incorporation into RBCs significantly compared to the control group, which received EPO alone; an incorporation of 5.16 ± 0.60% in the GM-CSF- and EPO-injected group compared to 8.98 ± 0.93% in the group injected with EPO alone ($P < 0.01$) (Table I). Mice that received only the vehicles incorporated 0.81% of injected ^{59}Fe into RBCs. To determine the dose of GM-CSF that was needed to cause significant suppression of ^{59}Fe incorporation, polycythemic mice were injected with various doses of GM-CSF together with 0.1 U EPO. Significant suppression was observed only when GM-CSF in doses of 1 μg or greater were injected together with EPO (Fig. 2). We also noted that a higher dose of EPO could abolish this suppressive effect of GM-CSF. Thus injection of 1 μg GM-CSF together with 0.1 U EPO produced a 42.5% suppression of iron incorporation, while no reduction in ^{59}Fe incorporation was observed when the same quantity of GM-CSF was injected together with 0.25 U EPO (Table I).

The timing of the GM-CSF injection with respect to EPO administration which results in significant suppression was investigated. One μg GM-CSF was injected at various times, from 24 hr prior to 24 hr after 0.1 U EPO. Injection of GM-CSF produced a significant inhibition when administered at intervals between 12 hr before or 6 hr after EPO injection (Fig. 3). For example, injection of GM-CSF 12 hr prior to EPO administration resulted in ^{59}Fe incorporation into circulating RBCs of 4.19 ± 1.01%, which was significantly lower than the 8.18 ± 0.95% in the control group that received EPO alone ($P < 0.01$). Injections of GM-CSF at intervals 24

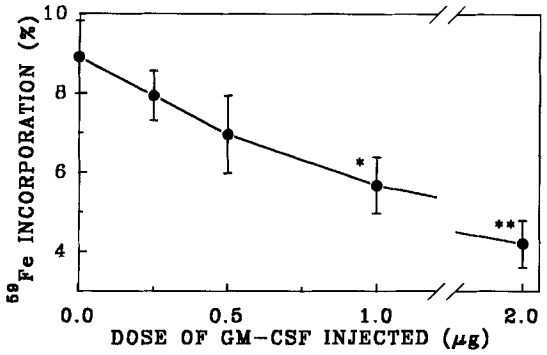

Fig. 2. ^{59}Fe incorporation into circulating RBCs (% of injected dose) of exhypoxic polycythemic mice, when 0.25–2 μg GM-CSF were injected simultaneously with 0.1 U EPO. Each point indicates mean ± SEM of at least 8 mice per group (values were collected in at least two separate experiments). *$P < 0.05$; **$P < 0.01$.

TABLE I. Effect of Injection of GM-CSF on ^{59}Fe Incorporation Into Circulating RBC†

Material injected	EPO injected (U)	% ^{59}Fe incorporation into RBCs
PBS (control)	0.1	8.98 ± 0.93*
1 μg GM-CSF	0.1	5.16 ± 0.60*
PBS (control)	0.25	15.00 ± 1.12
1 μg GM-CSF	0.25	15.61 ± 1.22

†Polycythemic mice were injected simultaneously with EPO (0.1 or 0.25 U) and GM-CSF or an equivalent volume of PBS 3 days after they were removed from the hypoxic chamber. Values are mean ± SEM of at least 6 mice per point (collected in two separate experiments).
*$P < 0.01$.

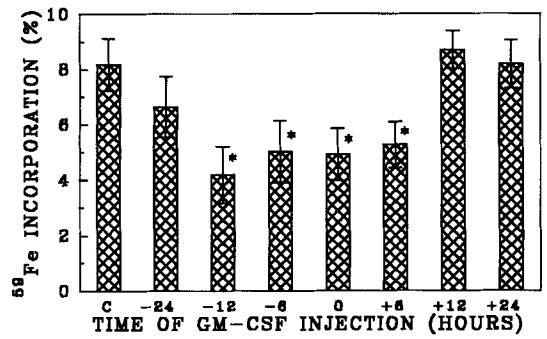

Fig. 3. Effect of injecting GM-CSF at various times, −24 hr–+24 hr of EPO injection, on ^{59}Fe incorporation into circulating RBCs of polycythemic mice. Each bar represents mean ± SEM of 6 mice per group (values were collected in at least two separate experiments). *$P < 0.05$.

hr prior to as well as 12 hr after EPO administration did not produce significant inhibitions.

We then investigated the effect of multiple injections of GM-CSF. In order to study this effect on EPO-induced

TABLE II. Effect of GM-CSF Injections on ^{59}Fe Incorporation Into Circulating RBCs of Exhypoxic Polycythemic Mice†

Material injected	Days of injection	% ^{59}Fe incorporation	% of control
PBS (control)	0, 1, 2, 3	9.56 ± 0.64	100.0
1 μg GM-CSF	0, 1, 2, 3	3.38 ± 0.35*	35.4
1 μg GM-CSF	0, 1, 2	4.73 ± 0.50*	49.5
1 μg GM-CSF	1, 2, 3	3.39 ± 0.52*	35.5
1 μg GM-CSF	0, 1	8.32 ± 1.02	87.0
1 μg GM-CSF	1, 2	5.42 ± 0.43*	56.7
1 μg GM-CSF	2, 3	4.46 ± 1.00*	46.7

†Injection intervals refer to the day the mice were taken out of the chamber in the case of exhypoxic polycythemic mice. Exhypoxic polycythemic mice were injected with 0.1 U EPO on day 3. All mice were injected with ^{59}Fe on day 5, and its incorporation into RBCs was measured on day 7. Values are mean ± SEM of at least 6 mice per interval (collected in two separate experiments).
*$P < 0.5$, compared to phosphate-buffered saline control.

erythroid cell formation, EPO was injected on the third day mice were out of the hypoxic chamber, and 1 μg GM-CSF was injected on that day as well as on prior days. Incorporation of ^{59}Fe into RBCs was significantly reduced (3.38 ± 0.35%) when the mice received four injections of 1 μg GM-CSF on days 0, 1, 2, and 3 after they came out of chamber, compared to 9.56 ± 0.64% in the control receiving vehicle only ($P < 0.05$, Table II). When the mice received two injections on days 0 and 1 after they were out of the chamber there was no suppression of ^{59}Fe incorporation. Furthermore, the most effective schedule that produced significant inhibition was when the GM-CSF injection was concurrent with EPO administration.

It is possible that the suppression of erythropoiesis observed in the case of endotoxin injection may be due to the production of GM-CSF [12]. To investigate this possibility, 250 μg of monoclonal anti-GM-CSF antibody were injected 30 min after 0.1 μg/mouse endotoxin and EPO administration. As shown in Figure 4, this injection did not abolish the effect of endotoxin upon iron incorporation. Injection of higher doses, of up to 1,000 μg of antibody, did not alter the suppression induced by endotoxin injection (data not shown). This further proved that the production of GM-CSF by endotoxin was not the cause of inhibition of iron incorporation in polycythemic mice. Another cytokine produced by the injection of endotoxin was TNFα [8]. In order to investigate whether the inhibition of iron incorporation into RBCs by the injection of endotoxin or GM-CSF was due to the production of TNFα, monoclonal anti-TNFα antibody was injected into the mice which received endotoxin or GM-CSF along with EPO. In an initial experiment we confirmed that optimum time of administration of anti-TNFα was 6 hr prior to injections of endotoxin or GM-CSF, and the optimum dose was 250 μg/mouse, as reported earlier for

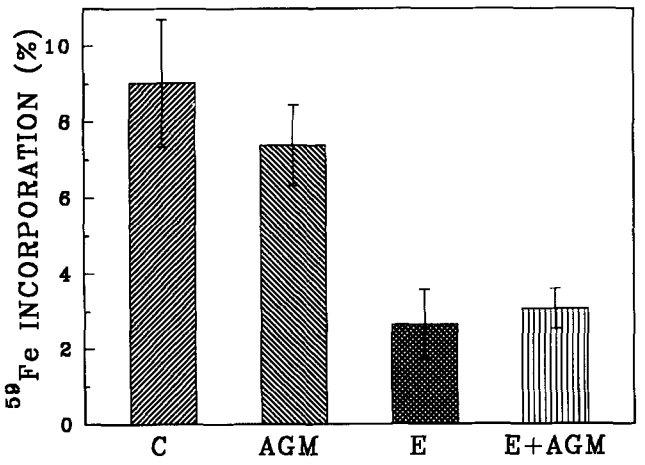

Fig. 4. Effect of injecting anti-GM-CSF antibody on ^{59}Fe incorporation into circulating RBCs of endotoxin and EPO-administered polycythemic mice. C, control; AGM, 250 μg anti-GM-CSF antibody; E, 0.1 μg endotoxin; E + AGM, endotoxin + anti-GM-CSF antibody. All mice received 0.1 U EPO. Each bar represents mean ± SEM of 6 mice per group (values were collected in at least two separate experiments).

TABLE III. Effect of anti-TNFα Antibody Injection 6 hr Prior to Simultaneous Injection of EPO and Endotoxin or GM-CSF on ^{59}Fe Incorporation Into Circulating RBCs of Exhypoxic Polycythemic Mice†

Group number	Materials injected and time of injection	% ^{59}Fe incorporation
1	PBS at −6 hr (control)	9.67 ± 0.69
2	250 μg anti-TNFα at −6 hr	8.39 ± 1.05
3	PBS at −6 hr + 0.2 μg endotoxin at 0 hr	1.34 ± 0.34
4	250 μg hamster IgG at −6 hr + 0.2 μg endotoxin at 0 hr	1.71 ± 0.79
5	250 μg anti-TNFα at −6 hr + 0.2 μg endotoxin at 0 hr	3.84 ± 0.84
6	PBS at −6 hr + 1 μg GM-CSF at 0 hr	4.70 ± 0.56
7	250 μg anti-TNFα at −6 hr + 1 μg GM-CSF at 0 hr	6.61 ± 0.68

†Injected materials and times of injections are indicated. In addition, all mice were injected with 0.1 U EPO at 0 hr. PBS, phosphate-buffered saline. Values are mean ± SEM of at least 12 mice per interval, accumulated in two separate experiments. $P < 0.02$, between groups 3 and 5; $P < 0.05$, between groups 6 and 7.

survival [8]. Injection of 250 μg anti-TNFα alone 6 hr prior to EPO did not inhibit iron incorporation (Table III). Injection of 0.2 μg endotoxin together with 0.1 U EPO reduced iron incorporation into RBCs to 15% of control. Injection of anti-TNFα in mice 6 hr prior to endotoxin along with EPO injections elevated the iron incorporation into RBCs significantly, compared to the group which received endotoxin and EPO without antibody ($P < 0.01$). Injection of hamster IgG equivalent to anti-TNFα 6 hr earlier than endotoxin and EPO injection did not produce this elevation. Suppression of iron incorporation into RBCs by the injection of GM-CSF was also lower in the group that received an anti-TNFα injection 6 hr earlier. These results indicate that the suppression of erythroid cell production, by the injection of GM-CSF as well as of endotoxin, was partly due to the production of TNFα in vivo.

DISCUSSION

The exhypoxic polycythemic mouse is a good model for the investigation of the late stages of erythroid cell proliferation. Injection of EPO generates a wave of erythroid cell formation, the amplitude of which is proportional to the dose of EPO injected. This model is similar to the in vitro culture of erythroid colony-forming units (CFU-E), where undifferentiated erythroid cells differentiate and form a colony of normoblasts which can be quantitated. Injection of any other material can enhance or suppress the level of erythroid cells formed, depending on the nature of the material injected. In this study only the effect on the formation of late stages of erythroid cells as indicated by the incorporation of ^{59}Fe into RBCs was investigated.

In an earlier study we showed that simultaneous administration of endotoxin together with EPO to exhypoxic polycythemic mice causes maximum suppression of erythroid proliferation, as compared to other intervals pre- or post-EPO injection [3]. Endotoxin itself does not cause this inhibition, since the addition of endotoxin to CFU-E culture is not suppressive. Even prior incubation with serum collected from mice injected with endotoxin 3 hr earlier has no effect on erythroid colony formation in vitro [3]. Since this serum contains a high concentration of GM-CSF [12], we can infer that GM-CSF does not affect erythroid cell development directly. From the results of the studies in which anti-GM-CSF antibody was injected after endotoxin and EPO administration, it can be concluded that the neutralization of GM-CSF cannot abolish the suppressive effect of endotoxin. This once again supports the inference that GM-CSF does not suppress erythroid proliferation directly. Metcalf et al. [13] showed that multiple injections of GM-CSF administered to normal mice cause a decrease in erythroid cell number in the marrow and an increase in the spleen. Injected GM-CSF has caused in an increase in myeloid cells in the marrow, and this raises the question as to whether this increase in marrow myeloid cells competitively directed some early erythroid progenitors into the myeloid cell line [14–16]. Our study indicates that the decrease in erythroid cell number in the marrow is not due to the direct effect of injected GM-CSF, but is mediated through the production of other factors.

The biological activity (ED_{50}) of the GM-CSF used in this study is 0.1 ng/ml, as determined by the dose-dependent granulocyte/macrophage colony formation from mu-

rine bone marrow cells (Upstate Biotechnology Inc. certificate of analysis sheet). We have confirmed this analysis in our laboratory. As shown earlier, in order to obtain a significant suppression of erythroid cell proliferation in polycythemic mice, we need to inject 1 μg of GM-CSF simultaneously with 0.1 U of EPO (i.e., 10,000 times the ED_{50}). Hence, a high dose of GM-CSF is necessary in order to observe inhibition, while a dose of 50 ng of endotoxin is sufficient to cause a significant suppression of erythroid proliferation. However, simultaneous injection of 1 μg of GM-CSF with a higher dose of EPO (0.25 U) is not inhibitory and can reverse the effect produced by GM-CSF. The time of injection of GM-CSF is also critical for the observed suppression of erythroid cell proliferation. Injection of GM-CSF must be 12 hr prior to or 6 hr after EPO administration. This indicates that GM-CSF does not act directly on the cells that proliferate and become hemoglobin-synthesizing cells in response to EPO administration, but rather through the production of other cytokines. The observation of no suppression of erythroid cell proliferation, when GM-CSF was injected at 12 hr or later (when the wave of erythroid cell production was maximal), indicates that the proliferation is unaffected by GM-CSF administration. As mentioned earlier, the dose of GM-CSF needed to inhibit erythroid cell proliferation in vivo is quite high. One of the reasons for this may be that the level of this growth factor in the circulation is not elevated enough by low doses to cause inhibition. Even multiple injections given over 2 days failed to show any additive effect. The second reason may be that, as discussed below, the production of other cytokines is triggered only by a high level of GM-CSF in the circulation.

Sheehan et al. [8] showed that injection of a lethal dose of endotoxin to mice can be overcome by the administration of 250 μg of monoclonal anti-TNFα antibody 6 hr earlier. This indicates that TNFα is the major cytokine causing this lethal effect. When we utilized this approach to investigate whether the suppression of erythropoiesis by endotoxin as well as GM-CSF could be altered by the injection of the same dose of anti-TNFα antibody, erythroid cell proliferation was higher in the groups that received antibody to TNFα 6 hr prior to endotoxin or GM-CSF administration, compared to those groups without antibody treatment. Thus in both cases anti-TNFα injections were beneficial, indicating the neutralization of TNFα in these cases. Our in vitro observation of neutralization of inhibition in erythroid culture containing GM-CSF by anti-TNFα antibody also supports this [7]. Thus we can arrive at the conclusion that the suppression of erythropoiesis by endotoxin as well as GM-CSF is partly due to TNFα produced by their injections. In both cases, however, ^{59}Fe incorporation into RBCs was not restored to the value of the control group that received antibody and EPO. This may have been due to the limited quantity of antibody injected, or to the need for several small injections of antibody to counteract TNFα produced at later hours. There is yet another possibility, that several other cytokines were produced by the injections, namely interferon γ and transforming growth factor β, both of which have been shown to suppress erythropoiesis [17,18].

This study has clearly shown that GM-CSF does not have any direct effect upon erythroid cells generated in polycythemic mice by the injection of EPO. Both endotoxin and GM-CSF suppress late-stage erythroid cells in vivo, partly by the production of TNFα. This information can help in treating anemia due to infection, inflammation, or any condition where the level of TNFα is elevated.

ACKNOWLEDGMENTS

This work was supported by the Arkansas EPSCoR Program, funded by the National Science Foundation, the Arkansas Science and Technology Authority, and the University of Arkansas for Medical Sciences, and by funds from the Department of Veterans Affairs.

REFERENCES

1. Fruhman GJ: Bacterial endotoxin: Effects on erythropoiesis. Blood 27:363, 1966.
2. Twentyman PR: The effects of repeated doses of bacterial endotoxin on erythropoiesis in the normal and splenectomized mouse. Br J Haematol 22:169, 1972.
3. Udupa KB, Reissmann KR: In vivo and in vitro effects of bacterial endotoxin on erythroid precursors (CFU-E and ERC) in the bone marrow of mice. J Lab Clin Med 89:278, 1977.
4. Niskanen E, Quesenberry P, Stohlman F Jr, Levin J, Ryan M: Colony-stimulating and inhibiting factors in sera from normal and endotoxin-treated CF_1 mice. Proc Soc Exp Biol Med 143:176, 1973.
5. Beutler B, Milsark IW, Cerami AC: Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 229:869, 1985.
6. Apte RN, Durum SK, Oppenheim JJ: Opioids modulate interleukin-1 production and secretion by bone-marrow macrophages. Immunol Lett 24:141, 1990.
7. Wang CQ, Udupa KB, Lipschitz DA: The role of macrophages in the regulation of erythroid colony growth in vitro. Blood 80:1702, 1992.
8. Sheehan KCF, Ruddle NH, Schreiber RD: Generation and characterization of hamster monoclonal antibodies that neutralize murine tumor necrosis factors. J Immunol 142:3884, 1989.
9. Reissmann KR, Udupa KB: Effect of erythropoietin on proliferation of erythropoietin-responsive cells. Cell Tissue Kinet 5:481, 1972.
10. Yoffey JM, Makin GS, Yates AK, Davis CJF, Griffiths DA, Waring IS: The discharge of granulocytes from guinea pig marrow in response to intravenous T.A.B. vaccine: A quantitative study. Ann NY Acad Sci 113:790, 1964.
11. Quesenberry PJ, Morley A, Miller M, Rickard K, Howard D, Stohlman F Jr: Effect of endotoxin on granulopoiesis and the in vitro colony-forming cell. Blood 41:391, 1973.
12. Quesenberry P, Halperin J, Ryan M, Stohlman F Jr: Tolerance to the granulocyte-releasing and colony-stimulating factor elevating effects of endotoxin. Blood 45:789, 1975.
13. Metcalf D, Begley CG, Williamson DJ, Nice EC, Lamarter JD, Mermod

J, Thatcher D, Schmidt A: Hematopoietic responses in mice injected with purified recombinant murine GM-CSF. Exp Hematol 15:1, 1987.
14. Van Zant G, Goldwasser E: Competition between erythropoietin and colony-stimulating factor for target cells in mouse marrow. Blood 53:946, 1979.
15. Steinberg HN, Handler ES, Handler EE: Assessment of erythropoietic and granulopoietic colony formation in an in vivo plasma clot diffusion chamber culture system. Blood 47:1041, 1976.
16. De Haan G, Engel C, Dontje B, Nijhof W, Loeffler M: Mutual inhibition of murine erythropoiesis and granulopoiesis during combined erythropoietin, granulocyte colony-stimulating factor, and stem cell factor administration: In vivo interaction and dose-response surfaces. Blood 84:4157, 1994.
17. Means RT, Krantz SB: Inhibition of human colony-forming units by interleukin-1 is mediated by gamma interferon. J Cell Physiol 150: 59, 1992.
18. Keller JR, Sing GK, Ellingsworth LR, Ruscetti SK, Ruscetti FW: Two forms of transforming growth factor-β are equally important selective growth inhibitors of early murine hematopoiesis. Ann NY Acad Sci 593:172, 1990.